THE EFFECTS OF REPLACING FETAL BOVINE SERUM WITH PLATELET RELEASATE ON THE CHARACTERIZATION OF EQUINE MESENCHYMAL STROMAL CELLS

An Undergraduate Research Scholars Thesis

by

CATHERINE CAPRIO and CAROLINE ANDREWS

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ABSTRACT

The Effects of Replacing Fetal Bovine Serum with Platelet Releasate on the Characterization of Equine Mesenchymal Stromal Cells

Catherine Caprio
Department of Biomedical Sciences and Veterinary Medicine
Texas A&M University

Caroline Andrews
Department of Biology
Texas A&M University

Research Advisor: Dr. Ashlee Watts
Department of Large Animal Clinical Sciences
Texas A&M University

Substituting fetal bovine serum (FBS) for platelet derivatives in mesenchymal stem cell (MSC) culture media is a current area of interest in orthopedic regenerative medicine. FBS, commonly used in *ex vivo* cell expansion, is a xenogen and can cause an immunological response in the subject after transplantation. Our objective was to determine if platelet releasate (PR) is a suitable replacement for FBS in the isolation and expansion of equine MSCs. MSCs were isolated from the raw bone marrow of five horses and plated with media containing either 10% FBS or 10% autologous PR. Cells were passaged three times once a sufficient confluency was reached, then cryopreserved. At each feeding, cells were photographed and counted, and morphology, debris, and confluence were recorded. Compared to cells grown in FBS, the MSCs grown in PR media had poorer morphology and showed signs of osteo-differentiation. Additionally, cells of the PR condition grew larger than those of FBS, which is an indication of

cell degradation. From this, we concluded that PR is not a suitable replacement for FBS in equine MSC cell expansion media.

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NOMENCLATURE

bFGF	Basic fibroblast growth factor
CFUs	Colony forming units
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
FBS	Fetal bovine serum
HBSS	Hank's balance salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
MEM	Minimum essential media
MSCs	Mesenchymal stromal cells
PL	Platelet lysate
PR	Platelet releasate
T75	Culture flasks
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Fetal bovine serum (FBS) is a media additive used to culture both human and equine MSCs . In 2008, the Committee for Medicinal Products for Human Use disapproved the use of FBS because it can potentially induce an immunological response in non-bovine cells due to its xenogeneic nature.[1] There is also concern for zoonotic pathogen transmission, lack of availability, and the potentially inhumane method of collecting FBS.[2] Therefore, finding a suitable replacement that is autologous, meaning from the same subject, and produces equal or improved cell cultures would be ideal.

Platelet lysate (PL) and platelet releasate (PR) are both platelet derivatives that have been studied as potential substitutes for FBS in humans. However, the methods in which growth factors are released differ between the two. PL is created through freezing and thawing cycles of platelets until the cells lyse, or rupture, releasing the growth factors from inside the cells. Previous experiments have shown that the repetitive thawing and freezing performed in obtaining PL could negatively affect the integrity of the necessary growth factors for cell expansion.[3]

Platelet releasate (PR), on the other hand, is not produced via freezing and thawing cycles. Instead, it is produced by activation of platelets.[4] Growth factors are released by means of calcium chloride ($CaCl_2$), a physiological stimulus that allows for preservation of the platelet cell membranes. Additionally, $CaCl_2$ allows for the removal of fibrinogen, which can alter the immunological properties of MSCs.[3] PR could be a more effective substitute than PL because release of growth factors has been shown to be increased using this method.[3]

PR could also be a more effective substitute for FBS, because FBS is derived from bovine blood, whereas autologous PR is derived from the same equine subject as the MSCs being isolated and expanded. The components of FBS have not been fully discovered or measured, while PR has more quantified ingredients. PR does not contain xenogens like FBS and should not produce immunological responses in the cells. In addition, PR has produced MSCs in humans that exhibit greater proliferation and improved characterization. Therefore, we hypothesized that PR will be an acceptable replacement for FBS, because it will produce similar or improved characterization in the cell culture of equine bone-marrow derived MSCs.

CHAPTER II

METHODS

To test our hypothesis, we used raw bone marrow that had been previously cryopreserved from five equine test subjects. After thawing the samples, the MSCs were isolated, expanded in media supplemented with FBS or PR, and frozen. The first condition was 10% FBS MSC isolation media, and the second condition was 10% autologous PR MSC isolation media. Both conditions were passaged from P0 to P3, then frozen after reaching 85% confluency at P3. Qualitative analysis was then performed through scoring photos of the MSCs at each stage of expansion.

Bone marrow plating from raw frozen cells

Raw bone marrow samples from five horses (Horses 1-5) were cryopreserved in liquid nitrogen. Cells were plated to both T75 flasks and a 10 cm CFU plates at a density of 3.5e6 total nucleated cells/cm^2 for each condition. After obtaining the necessary number of samples from the cryotank, cryovials were thawed slowly in a 37°C water bath for three minutes.

Under the fume hood, 1 mL of DPBS was slowly added to each cryovial. After five minutes, the contents of each cryovial was slowly transferred to a 50-mL conical tube in at least 20 mL of DPBS. A 100 µL sample was removed and counted under a UV microscope and cell counting solution, which contained fluorescein diacetate and propidium iodide. The DPBS suspension was pelleted through centrifugation for five minutes (300g), and the supernatant was aspirated. The cell pellet was resuspended in an appropriate amount of DPBS, and the suspension was evenly divided between two 50-mL conical tubes: one labeled FBS and the other labeled PR.

Both conical tubes were centrifuged for five minutes, and the supernatants of each were aspirated. The FBS-designated pellet was resuspended in the appropriate amount of 10% FBS MSC isolation media for the desired density, and the PR-designated pellet was resuspended in the appropriate amount of 10% autologous PR MSC isolation media. Twenty million cells were plated to a 10cm CFU plate, and 250 million cells were plated to a T75 flask.

MSC maintenance

Media was prepared from serum-free MSC isolation media (500 mL 1 g/dl glucose DMEM + 12.5 mL HEPES buffer + 5μL bFGF + 5μL anti-anti). Ingredients were warmed in 32-35°C water bath and combined in the original DMEM bottle. The contents were mixed by inverting the bottle several times, then sterile filtered into a sterile glass tissue culture media bottle. Media was stored at -20°C, and FBS and PR were added upon thawing as needed to produce 10% by volume solutions of each.

Each T75 flask and 10 cm dish was examined under a microscope for cell confluence, cell morphology, and debris three times per week. After taking photographs, media was aspirated and replaced with new respective media for each dish and flask. Once cell confluency reached approximately 70%, MSCs were passaged to a new cell culture flask.

Passaging MSCs

When the MSCs reached at least 70% confluency, the flasks and plates were examined, scored for morphology and debris, then passaged to a new flask. The flasks for each of the two conditions were rinsed first with HBSS. Then 1x trypsin was added to lift cells from plastic, and flasks were incubated for five minutes. The flasks were tapped and rocked to fully detach the

cells from the plastic. Trypsin was inactivated using 10% HyClone equine serum + HBSS. The cells were then aspirated, aliquoted into two 50-mL conical tubes, and pelleted through centrifugation. The cell pellets were resuspended in DPBS, a 100 µL sample from each was removed for cell counting, and the cells were pelleted again. The pellets were resuspended in either FBS or PR, keeping the conditions consistent, and replated at 5,000-7,000 cells/cm^2. This protocol was repeated until the cells had been passaged three times, from P0 to P3, then cryopreserved.

On the same day as the first passage, the 10 cm plates were fixed and stained to identify CFUs. First, the media was aspirated and the plates were rinsed twice with 1x PBS. Then, crystal violet was pipetted onto each of the plates and allowed to stain for ten minutes. After staining, the plates were rinsed with deionized water and allowed to dry overnight. CFUs were counted and recorded, and the plates were saved.

Cryopreservation for *in vivo* use

When the MSCs reached at least 85% confluency at P3, they were morphologically scored and then frozen for later use. The flasks were rinsed first with HBSS, then with 1x trypsin, and incubated for five minutes. After rocking the flasks to detach the cells from the surface, trypsin was inactivated with 10% HyClone equine serum and HBSS. The cells were then aspirated and consolidated into one 50-mL conical, keeping conditions separate. The cells were pelleted through centrifugation and resuspended in DPBS. A 100 µL sample was taken for cell counting, then the cells were pelleted and rinsed with MEM. After pelleting, the supernatant was aspirated and the pellet was gently resuspended in 1 mL of cryopreservation media per 10 million cells. Cryopreservation media was made using 95% autologous or allogeneic serum and

5% DMSO. After the cells were resuspended in the cryopreservation media, they were aliquoted into cryovials (10e6 cells per vial) and placed in the -80ºC freezer. After at least 24 hours, the cryovials were transferred to liquid nitrogen.

Qualitative analysis

Before each passage, the MSCs were photographed and scored based on morphology, debris, and confluency. Morphology was scored using a four-point scale, with 4 as excellent and 1 as poor. Excellent morphology is characterized by a long, spindle shape with defined nuclei and few air pockets within the cell. Poor morphology is characterized by a flat, polygonal shape with decaying nuclei and numerous air pockets. Some MSCs exhibited a jagged morphology with numerous cell projections. When this occurs, the cells have differentiated into osteocytes and are no longer MSCs, thus earning a score of 0.

The approximate cell length of the MSCs was also measured at each passage, based on 200 μm scale. This tracked cellular length as passages continued and allowed for comparisons between the two conditions.

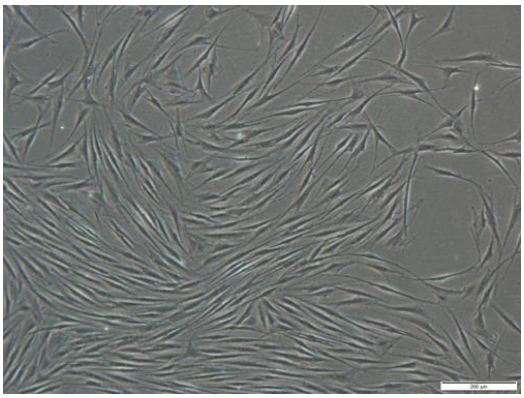
CHAPTER III

RESULTS

Morphological scoring

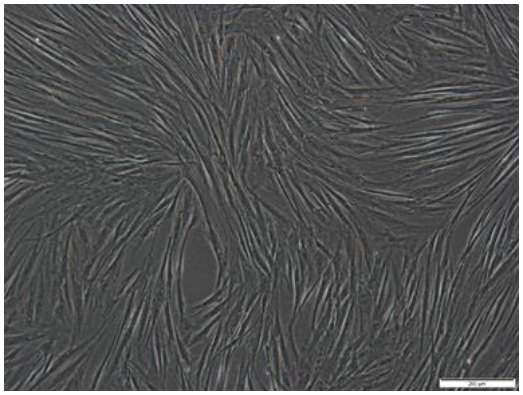
Cell morphology was scored according to a scale where 4 was excellent, 3 was good, 2 was fair, and 1 was poor. A score of 0 was given if there were signs of osteogenesis, indicating differentiation. Figure 1A shows excellent morphology in which cells are long and spindle-shaped, containing few vacuoles. Figure 1B shows good morphology in which cells were still spindle-shaped with a small number of vacuoles, but showed beginning signs of degradation. For fair morphology, as shown in Figure 1C, cells were not as extended, starting to take on a polygonal shape and showing jagged edges. Cells with poor morphology, as shown in Figure 1D were flat and kite-shaped, with more extended jagged edges. The osteocytes shown in Figure 1E were flat, kite-shaped, and had extensive networks of projections interacting with other cells.

A



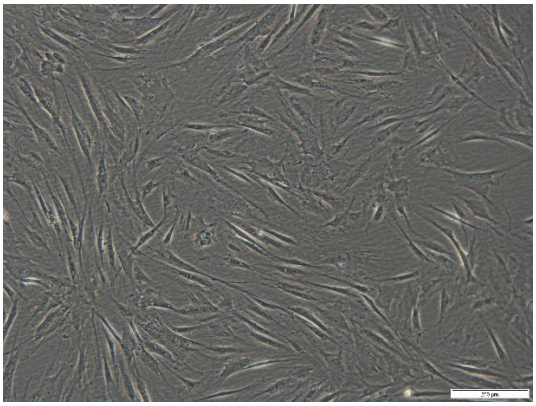
4—Excellent

B



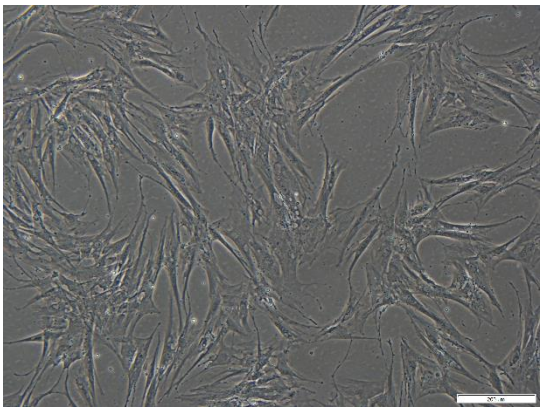
3—Good

C



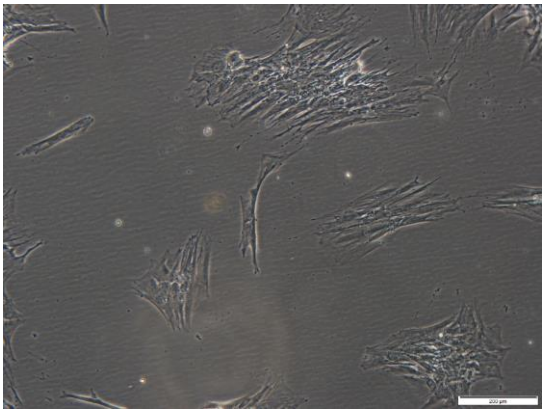
2—Fair

D



1—Poor

E



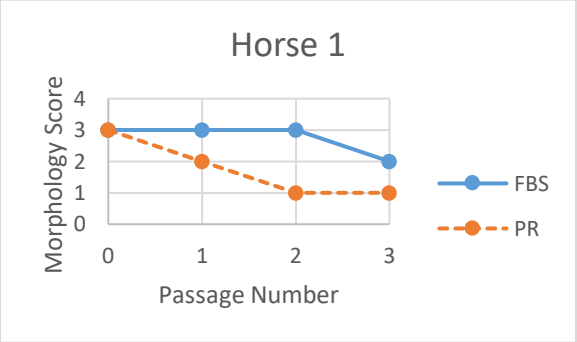
0—Osteogenesis

Figure 1. Morphology score. *Figure 1 shows examples of cellular morphology at each point in the scale, from excellent to poor to osteogenesis.*

MSC morphologies were scored before each passage for both FBS and PR conditions based on the above scale. Figure 2 shows that at P0, the two conditions are consistently equal in morphology. As the passages progressed in every horse, PR cells degraded in morphology and were scored as fair or poor by P2. Figures 2C and 2E show PR cells that underwent osteogenesis before P3. Cells grown in FBS consistently exhibited good or excellent morphology, with the exceptions of fair morphology displayed in Figures 2A and 2D. Figure 2C does not have a score for FBS at P0, as we were unable to photograph cells for that passage due to computer issues.

Figure 2D does not have a score for PR at P3 because our reserve of PR media was depleted during P2, rendering us unable to expand the cells further.

A



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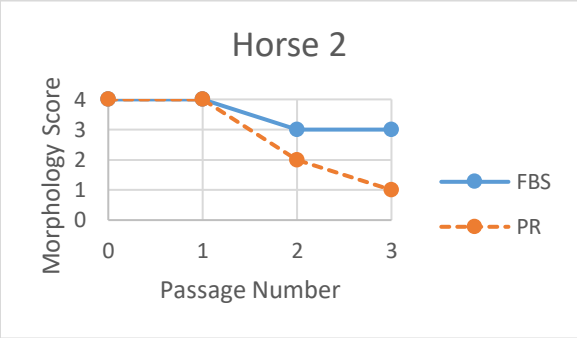

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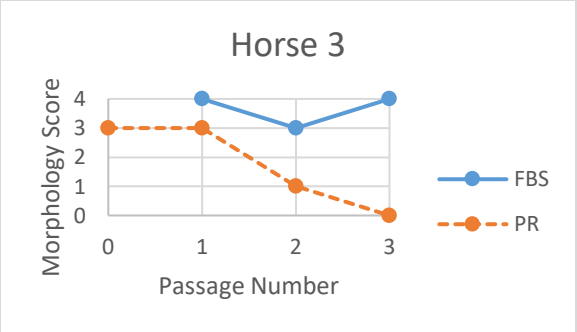

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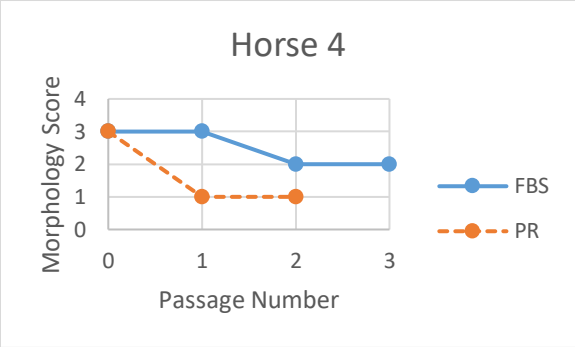

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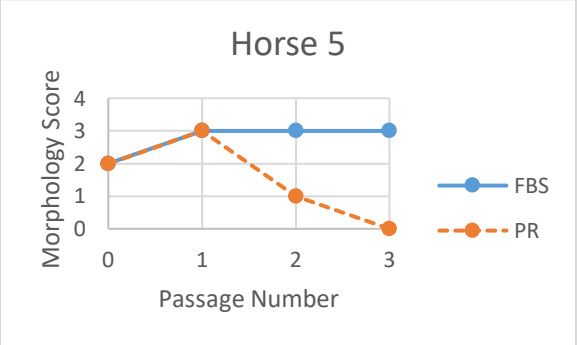
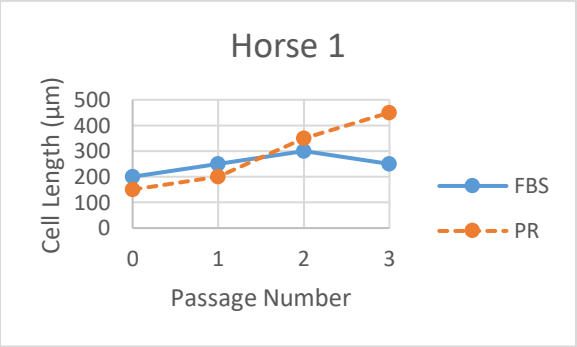


Figure 2. Morphology score vs. passage number. *Figure 2 shows the progression of cellular morphology for FBS and PR over multiple passages in five different horses.*

Cell length comparison

Cell length was measured before each passage for both FBS and PR conditions. As shown in Figure 3, cells lengths increased with each passage for both FBS and PR; however, PR cell length increased more than FBS cell length after passage 1 in every subject except Horse 2, which showed similar growth per passage for both conditions. PR cells from Horse 5 were consistent with this trend in that they were longer than FBS cells, however their size was maintained between P2 and P3 while FBS cells from Horse 5 decreased in length between passages 2 and 3.

A



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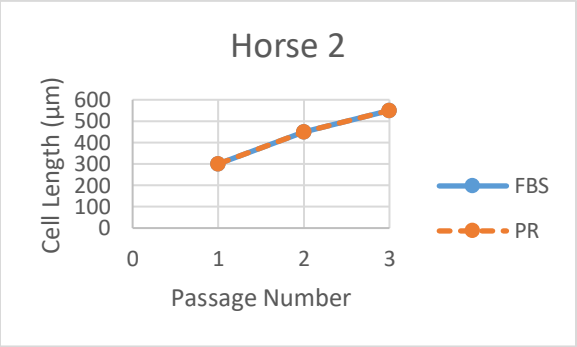

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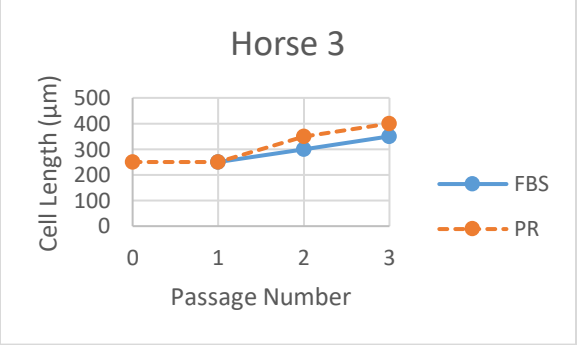

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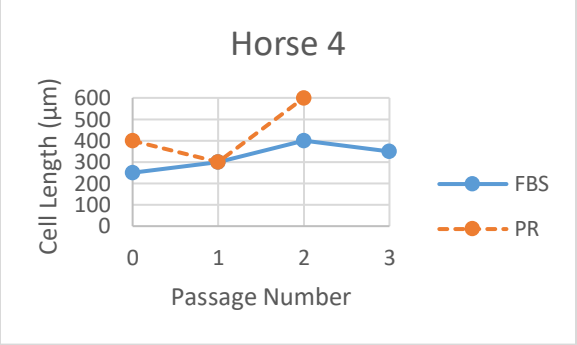

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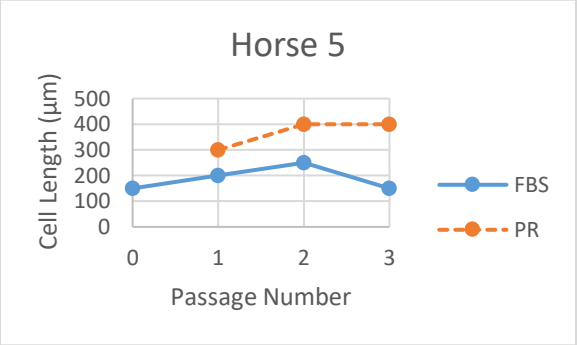

Figure 3. Cell length vs. passage number. *Figure 3 shows the progression of cellular length for FBS and PR over multiple passages in five different horses.*

CHAPTER IV

CONCLUSION

In conclusion, our hypothesis was not supported and we determined that platelet releasate is not a suitable substitute for fetal bovine serum. Equine bone marrow-derived MSCs exhibited excellent or good characterization when grown in 10% FBS media. MSCs grown in 10% PR media showed an excellent or good morphology in earlier passages, but deteriorating morphology in later passages. MSCs grown in both PR and FBS grew larger with each passage, which is typical with subsequent passages as cells degrade.[5] However, cells grown in PR grew larger in size than those grown in FBS, signaling greater degradation. Based on our results, the MSCs cultured in PR showed poorer morphology and greater cell size than those cultured in FBS.

Morphology score

MSCs at low passages are elongated, small, and spindle-shaped cells.[5, 6] MSCs at high passages are larger, flatter, and more polygonal, indicating the decay of differentiation potential.[5, 6, 7] A definitive sign of cellular degradation is the number of vacuoles observed within the cell. The presence of vacuoles within MSCs indicates a more mature stem cell that is reaching the end of its *in vitro* functionality.[7, 8]

Therefore, we created a scale that scored elongated, spindle-shaped MSCs with few vacuoles as a 4, meaning excellent morphology. Flat, polygonal, kite-shaped MSCs with many vacuoles were scored as a 1, meaning poor morphology. Some of the MSCs grown in PR at later passages had a jagged appearance with multiple cellular proliferations. These proliferations and

intricate cellular interactions are more associated with osteocytes rather than MSCs.[9] Cells that exhibited the characteristics of osteocytes were scored as a 0, because they no longer had the morphology of MSCs. To confirm osteogenesis, we would either need to stain with Alizarin Red or conduct flow cytometry.

Species difference

Our experiment was the first to examine the effects of platelet releasate on equine MSCs. However, other studies involving PR have been successfully conducted with human cells. In humans, PR has been proven to be a viable substitute for FBS, yielding increased cell proliferation and excellent characterization. A human study culturing bone-marrow derived MSCs showed that PR was comparable to FBS in MSC isolation and expansion.[10] However, the PR used in this experiment was not activated by thrombin to avoid any immunological responses due to equine thrombin not being commercially available. There may be other factors at play having to do with the differences between horse and human blood.

Horse blood contains more fibrinogen than human blood due to their herbivorous diet. Fibrinogen is a coagulation factor that has to be removed to prevent clot formation in cell culture. It also increases the adhesion of human natural killer cells that attack the bone marrow-derived MSCs, promoting inflammatory responses.[11] Also, equine platelets are smaller compared to human platelets. Equine plasma is also more viscous than human plasma, and contains more pigments like bilirubin and carotene.[12] These species differences in the blood may have affected PR to the point where experiments regarding human PR show vastly different results than our experiment involving horse PR.

Serum concentration

Although 10% serum proved to be a suitable amount for MSC expansion when the serum was FBS, a different percentage may be better suited for PR. Previous experiments testing platelet lysate (PL) as an alternative to fetal bovine serum have shown that varying the concentration of PL produces a bell-shaped curve response of cell proliferation of equine mesenchymal stem cells. Seven different concentrations of FBS and PL ranging from 5% to 60% were tested. While MSCs grown under the FBS conditions continued to show a consistent positive correlation with increasing serum concentration, cells grown in PL had an optimum growth range in concentrations between 20% and 40%.[13]

Although platelet lysate and platelet releasate are both platelet derivatives, they differ in their preparation methods and as a result, levels of platelet-derived growth factors. At concentrations of 5, 7.5 and 10%, PR overall contained higher concentrations of PDGFs resulting in MSCs with higher proliferation than PL.[3] Cells expanded in both PR and PL maintained MSC characterization; however, FBS still showed superior characterization over the platelet derivatives.[3] Both of these serums were derived from humans, so similar experiments should be done with equine MSCs to know whether or not their results would be consistent. Although the aforementioned experiment showed that a concentration of 20-40% PL in growth media is optimum, the latter found that PR might inherently contain more PDGF than PL, so a lower percentage of PR might be optimal. Due to the variability in literature of what the optimum platelet derivative percentage is, it would be beneficial to expand equine MSCs in media with varying percentages of PR above and below 10%.

Pooling

Although varying the concentration of platelet releasate in the expansion media could be beneficial to finding the optimum percentage, this is under the assumption that the concentration of growth factors in different preparations of PR is consistent. Unfortunately, this is not the case. Even when a single donor and a standardized activation method is used, platelet concentrations are vary among different batches of PR. Single-donor pooling has shown to reduce some of this variability in human PR. Results showed that a minimum of 16 single-donor batches should be pooled to reduce the percent coefficient of variation (CV) below a defined threshold of 20%.[14]

No pooling was used in preparation of PR for this project. This may have contributed to the variability in morphology and size among PR-conditioned MSCs seen at each passage. PR preparations made from pooling 16 or more batches of PR from a single-donor equine subject may reduce variability seen in the characterization of that subject's MSCs, and consequently, provide consistency in levels of growth factors when testing for the optimum percentage of PR in complete expansion media.[14]

Even though our hypothesis was not supported, using different concentrations of PR or pooling PR from multiple donors may improve results. Equine bone marrow-derived MSCs exhibited diminishing morphology when expanded in unpooled 10% PR media. If PR is researched further, it could still be a viable substitute for FBS in the expansion of equine MSCs.

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